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Determination of Monoterpene Hydrocarbons and Alcohols in *Majorana hortensis* Moench by Micellar Electrokinetic Capillary Chromatographic

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Micellar electrokinetic capillary chromatography was used to determine the essential oils obtained by steam distillation of different samples of marjoram (*Majorana hortensis* Moench) dried leaves and flowers. The electrophoretic method consisted of a running buffer of 10 mM NaH_2PO_4, 6 mM $Na_2B_4O_7$, 50 mM SDS, 7 mM γ-cyclodextrin, and 10% acetonitrile, adjusted to pH 8.0 by the addition of 0.1 M H_3PO_4. The following monoterpene hydrocarbons and alcohol compounds were extracted from real samples and determined by the method proposed: α-pinene, γ-terpinene, α-terpinene, terpinolene, *p*-cymene, linalool, α-terpineol, and terpinen-4-ol. The most prominent component of dried leaves, flowers, and commercial samples was terpinen-4-ol in four of the samples analyzed; only in one sample was α-terpineol present as the major compound.

KEYWORDS: ***Majorana hortensis* Moench; essential oil; micellar electrokinetic capillary chromatography; monoterpene hydrocabons and alcohols**

INTRODUCTION

The vernacular term marjoram comprises several aromatic Labiatae herbs belonging to different species. *Origanum majorana* L. (syn. *Majorana hortensis* Moench., *M. vulgaris* Miller) is the type of marjoram most well-known. This type of sample is a native of Cyprus and southern Turkey. It is cultivated extensively as sweet marjoram, an annual herb, in several areas of Europe, Africa, America, and Asia. The dried leaves of sweet marjoram are widely used by the food industry as flavoring agents for dressings and soups and in the formulation of vermouth and bitters, among others (*1*−*3*). The essential (volatile) oil of sweet marjoram has been known since antiquity due to its biological activities, such as antibacterial, antifungal, and antioxidant properties. For all of these reasons, the flavor composition of cultivated marjoram has been investigated in recent years (*4*, *5*). Nykänen (*6*) has demonstrated that the aroma composition varies with the origin of plants and many other factors. These authors found essential oils of marjoram with high contents of monoterpene alcohols and other phenols. The term essential oil implicates by definition the method of preparation, namely, the separation of volatile substances by distillation at atmospheric pressure and elevated temperature (*7*). In some oils, terpinen-4-ol was the major component, alone or together with other monoterpene alcohols such as *cis*- and *trans*-sabinene hydrate and α-terpineol. High contents of carvacrol (65%) were found in marjoram oil by Sarer et al. (*3*), whereas Nykänen (*6*) reported a type of marjoram oil with high levels of thymol (up to 47%).

Other methods of distillation, such as simultaneous steam distillation−extraction (SDE) and supercritical fluid extraction (SFE), can also produce high-quality essential oils from herbaceous materials (*8*, *9*). Capillary gas chromatography with mass spectrometry was widely used to analyze the composition of essential oils (*3*−*12*). High-performance liquid chromatography (HPLC) is widely employed for the determination of antioxidants present in extracts from plants (*13*, *14*). Nowadays, micellar electrokinetic capillary chromatographic (MEKC) is also used for the separation and quantification of these hydrophobic antioxidants, which can be present as different enantiomeric compounds (*15*, *16*). MEKC is a mode of capillary electrophoresis based on different partitioning of the analytes between the micelle and aqueous phase. The addition of modifiers was necessary to separate compounds that migrated at the same velocity as the micelle. For the separation of this type of analyte, cyclodextrins (α, β, and γ) and organic solvents such as methanol and acetonitrile are generally employed.

Determination of the chemotype in marjoram samples is very important for the industry and in some biomedical research works. Monoterpene alcohols are the main aroma components

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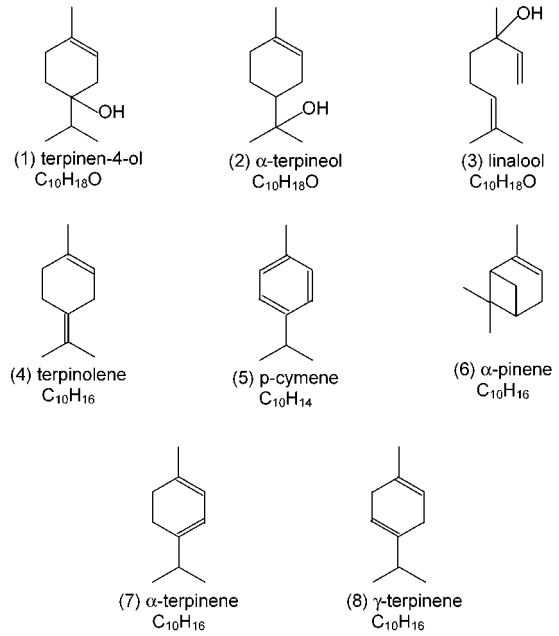

Figure 1. Chemical structures of the monoterpene alcohols and hydrocarbons used in this study.

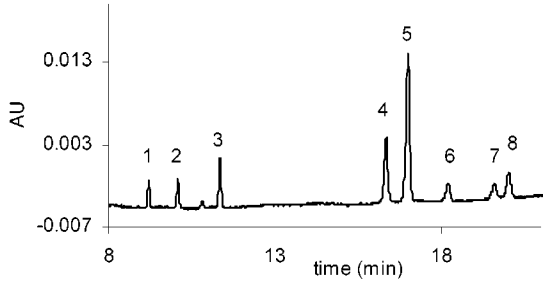

Figure 2. Electropherogram of eight monoterpene alcohols and hydrocarbons. Peaks: (1) terpinen-4-ol, (2) α-terpineol, (3) linalool, (4) terpinolene, (5) *p*-cymene, (6) (+)-α-pinene, (7) α-terpinene, and (8) γ-terpinene. Conditions: buffer, 10 mM phosphate, 6 mM borate, 50 mM SDS, 7 mM γ-CD, and 10% acetonitrile; pH 8.0; fused-silica capillary, 40 cm × 75 μm i.d.; applied voltage, 20 kV; detection, 200 nm; temperature, 25 °C.

of fresh marjoram. The identification of the compounds studied in this work could be used to classify these samples according to their geographical origin and to detect fraud in commercial samples.

In this work, steam distillation was used for the extraction of essential oil from dried leaves and flowers of *Majorana hortensis* Moench. Different samples cultivated in the south of Brazil and in Spain were used. MEKC was used for the separation and quantification of eight monoterpene hydrocarbons and alcohols present in these types of samples. These compounds were chosen because they are a majority in these types of samples.

A review of the literature found no references dealing with the determination of these compounds in marjoram samples by MEKC. The method previously developed by the authors (*17*) for the separation and quantification of seven monoterpene hydrocarbons was adapted to include monoterpene alcohols.

EXPERIMENTAL PROCEDURES

Samples. Seeds of *M. hortensis* Moench were acquired from The Netherlands (M1), Denmark (M2), and Germany (M3), and they were cultivated under the control of the Agricultural Experimental Station (FEPAGRO) in southern Brazil. Voucher specimens were identified and deposited at the herbarium of FEPAGRO. Fresh leaves and flowers from these plants dried at room temperature for 3 weeks were used in this study. Commercially available marjoram samples were purchased in markets, one from Sevilla in southern Spain (M4) and another from Soria, in northern Spain (M5).

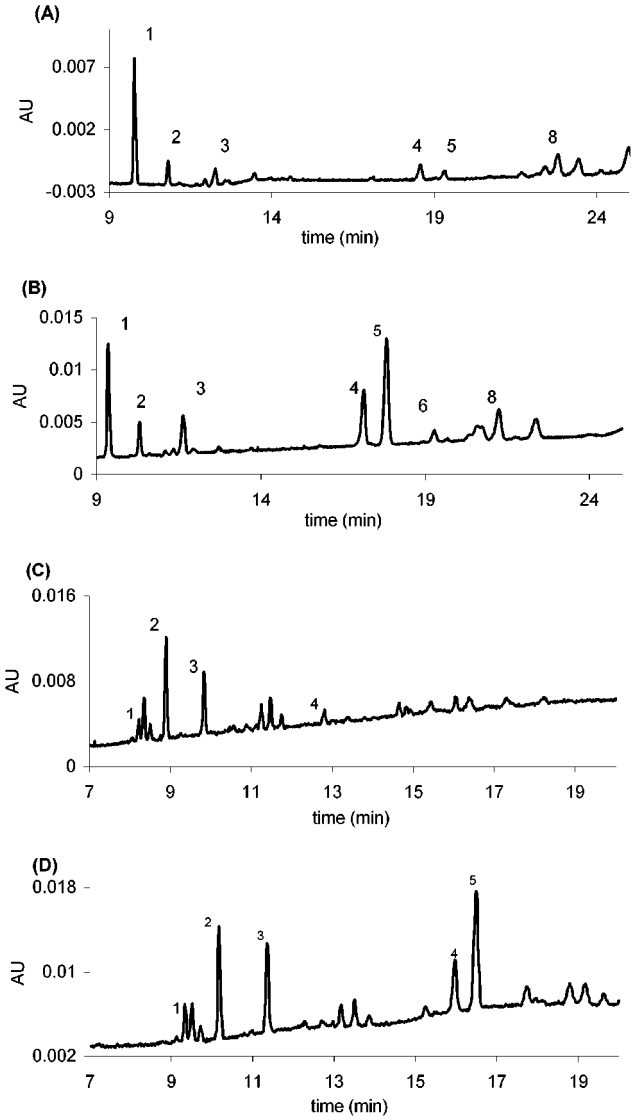

Figure 3. Electropherograms of eight monoterpene alcohols and hydrocarbons in (A) essential oil of marjoram M2, (B) essential oil of marjoram M2 spiked with 5 μg mL^{-1} of each monoterpene alcohol and hydrocarbon, (C) essential oil of marjoram M4, and (D) essential oil of marjoram M4 spiked with 5 μg mL^{-1} of each monoterpene alcohol and hydrocarbon. Peaks: (1) terpinen-4-ol, (2) α-terpineol, (3) linalool, (4) terpinolene, (5) *p*-cymene, (6) (+)-α-pinene, (7) α-terpinene, and (8) γ-terpinene. Conditions: see **Figure 2**.

Standards. Monoterpene alcohols and hydrocarbons (see **Figure 1**) such as terpinen-4-ol, α-terpineol, linalool, terpinolene, *p*-cymene, α-terpinene, and γ-terpinene were obtained from Fluka, and (+)-α-pinene was from Sigma. γ-Cyclodextrin was obtained from Fluka, and sodium dodecyl sulfate (SDS) was obtained from Sigma. NaH_2PO_4 and $Na_2B_4O_7$ were obtained from Aldrich. Stock solutions (1000 μg mL^{-1}) of each monoterpene were made up in methanol, and all were stored in the refrigerator.

Isolation of Essential Oil. Fresh and dried leaves and commercially available plant material (40 g) were subjected to hydrodistillation until there was no significant increase in the volume of the collected oil (4.5 h) in a Clevenger-type apparatus (*18*−*20*). The isolated oils were dried over anhydrous sodium sulfate and stored under N_2 in a sealed

Table 1. Figures of Merit of the Monoterpene Hydrocarbons and Alcohols[a]

analyte	$y = a + bx$	$S_{y/x}$	r	R^2	RSD area (%)	RSD time (%)	LOD	LOQ
terpinen-4-ol	$a = 0.52 \times 10^{-2} \pm 0.73 \times 10^{-2}$ $b = 2.32 \times 10^{-2} \pm 0.06 \times 10^{-2}$	0.015	0.996	99.23	5.5	2.3	0.94	3.14
α-terpineol	$a = 0.26 \times 10^{-2} \pm 1.06 \times 10^{-2}$ $b = 3.01 \times 10^{-2} \pm 0.09 \times 10^{-2}$	0.022	0.995	99.04	5.4	2.2	1.06	3.54
linalool	$a = -0.37 \times 10^{-2} \pm 0.17 \times 10^{-2}$ $b = 5.76 \times 10^{-2} \pm 0.15 \times 10^{-2}$	0.035	0.997	99.34	8.1	2.9	0.09	0.30
terpinolene	$a = -14.96 \times 10^{-2} \pm 5.36 \times 10^{-2}$ $b = 14.79 \times 10^{-2} \pm 0.48 \times 10^{-2}$	0.112	0.995	98.97	8.0	2.8	1.09	3.62
p-cymene	$a = -16.92 \times 10^{-2} \pm 11.68 \times 10^{-2}$ $b = 33.27 \times 10^{-2} \pm 1.04 \times 10^{-2}$	0.243	0.995	99.04	7.6	2.7	1.05	3.51
α-pinene	$a = -6.90\ 10^{-2} \pm 1.46\ 10^{-2}$ $b = 4.71 \times 10^{-2} \pm 0.13 \times 10^{-2}$	0.030	0.994	99.25	8.0	2.8	0.93	3.10
α-terpinene	$a = -4.30 \times 10^{-2} \pm 1.40 \times 10^{-2}$ $b = 4.38 \times 10^{-2} \pm 0.12 \times 10^{-2}$	0.029	0.996	99.19	6.3	2.5	0.96	3.20
γ-terpinene	$a = -8.72 \times 10^{-2} \pm 3.54 \times 10^{-2}$ $b = 8.18 \times 10^{-2} \pm 0.31 \times 10^{-2}$	0.074	0.993	98.54	7.5	2.7	1.30	4.33

[a] a = intercept; b = slope; r = correlation coefficient; $S_{y/x}$ = standard deviation of residuals; R^2 = curve-fitting level (%) obtained by ANOVA for the validation of the method; RSD = relative standard deviation of the peak area and electrophoretic migration time values; LOD = limit of detection; LOQ = limit of quantification. Concentration, LOD, and LOQ are expressed in μg mL^{-1}. Buffer: 10 mM NaH_2PO_4, 6 mM $Na_2B_4O_7$, 50 mM SDS, 7 mM γ-CD, and 10% acetonitrile; pH 8.0; 20 kV; 25 °C; 5 s hydrodynamic injection; 200 nm.

vial until required. Stock solutions (1000 μg mL^{-1}) of each essential oil were dissolved in methanol, and all were stored in the refrigerator.

Apparatus and Electrophoretic Separation Conditions. A Beckman P/ACE 5500 capillary electrophoresis system equipped with a diode array detector and a System Gold data station were used in this study. A fused-silica capillary column, 75 μm i.d. with an effective length (between inlet and detector) of 40 cm (total length = 47 cm), was used for the separation of the analytes.

All of the separations were performed at a temperature of 25 °C, and the applied voltage was 20 kV (average current = 75 μA). The samples were hydrostatically (5 s) introduced into the anodic end of the capillary. Detection was recorded at 200 nm. The electrolyte consisted of 10 mM NaH_2PO_4, 6 mM $Na_2B_4O_7$, 50 mM SDS, 7 mM γ-cyclodextrin, and 10% acetonitrile, adjusted to pH 8.0 by the addition of 0.1 M H_3PO_4. Daily, the capillary was rinsed for 10 min with deionized water, for 10 min with 0.1 M NaOH, for 5 min with deionized water, and for 15 min with running buffer. The flush between runs was of 1 min with deionized water, 0.1 M NaOH (2 min), deionized water (2 min), and buffer (3 min). These steps were necessary to prevent the adsorption of monoterpenes on the wall of the capillary. New capillaries were conditioned for 5 min with deionized water, for 10 min with 1 M HCl, for 5 min with deionized water, for 10 min with 0.1 M NaOH, for 5 min with deionized water, and for 20 min with running buffer. Working standard solutions of mixtures were obtained by appropriate dilution in water of the stock standard solutions, and they were prepared fresh daily.

RESULTS AND DISCUSSION

Extraction of Essential Oils. Leaves and flowers of five samples of marjoram (M1, The Netherlands; M2, Denmark; M3, Germany; M4, Sevilla, Spain; and M5, Soria, Spain) were air-dried and subjected to hydrodistillation using a Clevenger apparatus to produce oils with a yield between 1.1 and 1.4% w/v (M1 = 1.2%, M2 = 1.2%, M3 = 1.4%, M4 = 1.2%, and M5 = 1.1%). These values were comparable with those reported in the literature (*2*, *21*).

Separation and Determination of Analytes. The first step was to test the separation of the eight monoterpenes by using standards. A good separation of the analytes was achieved following the electrophoretic conditions previously reported by the authors (*17*). The method was evaluated by means of calibration curves and reproducibility experiments and by using the buffer and conditions mentioned under Experimental Procedures. **Table 1** shows the corresponding regression equations and other parameters obtained for all monoterpenes electrophoretically separated in a concentration range between 1 and 15 μg mL^{-1}. Standard deviation of residuals ($S_{y/x}$) and curve-fitting level (R^2) were obtained by analysis of variance (ANOVA) during the validation of the calibration model. Values between 0.24 and 0.01 were obtained for $S_{y/x}$ in all cases, whereas R^2 was always >98.54% for the analytes determined. The limit of detection (LOD) was calculated by using 3 times the standard deviation of the intercept divided by the slope, whereas the limit of quantification (LOQ) was calculated from 10 times the standard deviation of the intercept divided by the slope. The proposed method allows the determination of monoterpenes at low concentration levels (LODs between 0.09 and 1.30 μg mL^{-1}). The LOQs obtained were between 0.3 and 4.33 μg mL^{-1} for all monoterpenes. Eleven replicates were performed on the standard solution (5 μg mL^{-1} for each monoterpene). In all cases the relative standard deviations (RSDs) for the retention times were <2.9% and <8.1% for the peak areas.

Analytical Applications to Real Samples. The proposed method was applied to the direct determination of eight analytes in five marjoram samples. Using the experimental conditions optimized in a previous work (*17*), optimum separation and quantification were obtained for monoterpene alcohols and hydrocarbons. **Figure 2** shows an electropherogram of eight monoterpenes at 10 μg mL^{-1}. The UV−visible spectra of all monoterpenes were very similar; α-pinene presents a spectrum slightly different because its structure is bicyclic, despite its being an isomer of the other monoterpene hydrocarbons in this study.

Solutions of each marjoram sample (M1−M5) were injected at various levels of concentration (10−500 μg mL^{-1}). A 100 μg mL^{-1} concentration was selected for the analysis of real samples due to good resolution achieved between the peaks

Table 2. Determination of the Analytes in Marjoram Samples Using the Proposed Method

analyte	real sample	concn (μg mL^{-1}) added	concn (μg mL^{-1}) found	recovery (%)	concn in real sample (μg mL^{-1})	real sample	concn (μg mL^{-1}) added	concn (μg mL^{-1}) found	recovery (%)	concn in real sample (μg mL^{-1})
terpinen-4-ol	marjoram 1	1	0.8	80.4	31.4	marjoram Sevilla	1	1.1	103.5	4.2
		3	3.2	107.2			3	2.9	96.8	
		5	5.5	109.1			5	5.1	100.8	
		10	11.0	110.1			10	10.3	103.4	
		15	15.7	104.7			15	15.6	103.8	
	marjoram 2	1	1.1	105.4	24.8	marjoram Soria	1	1.1	111.1	20.0
		3	2.6	87.0			3	3.1	103.8	
		5	4.8	95.9			5	5.1	101.6	
		10	10.5	104.5			10	10.5	105.1	
		15	15.5	103.0			15	15.5	103.3	
	marjoram 3	1	0.9	97.8	34.5					
		3	3.2	108.2						
		5	5.1	101.3						
		10	10.4	104.1						
		15	15.0	100.0						
γ-terpinene	marjoram 2	1	0.9	94.4	6.4	marjoram Soria	1	1.0	100.0	10.4
		3	3.2	105.5			3	3.1	103.1	
		5	5.2	104.1			5	5.4	108.7	
		10	10.1	101.1			10	9.9	99.1	
		15	14.8	98.5			15	15.7	104.4	
	marjoram 3	1	1.1	108.5	19.8					
		3	3.0	100.0						
		5	5.2	104.6						
		10	10.6	106.2						
		15	15.0	100.0						
linalool	marjoram 1	1	1.0	100.0	3.4	marjoram Sevilla	1	0.9	96.3	5.1
		3	2.9	97.5			3	2.8	95.0	
		5	5.2	104.4			5	5.1	100.7	
		10	10.1	100.6			10	10.2	102.0	
		15	15.0	100.0			15	15.8	105.2	
	marjoram 2	1	1.0	100.0	4.5	marjoram Soria	1	0.9	98.9	3.6
		3	3.3	108.5			3	3.1	101.4	
		5	5.4	107.4			5	5.1	101.6	
		10	10.6	105.7			10	10.1	100.8	
		15	14.9	99.5			15	14.9	99.1	
	marjoram 3	1	0.8	81.4	3.5					
		3	3.1	103.7						
		5	5.6	112.3						
		10	9.3	93.1						
		15	15.5	103.6						
α-pinene	marjoram 2	1	1.0	100.0	ND[a]	marjoram Soria	1	0.8	82.4	1.0
		3	3.1	101.4			3	3.1	103.1	
		5	5.1	101.2			5	5.4	108.7	
		10	9.9	99.5			10	10.1	101.0	
		15	15.1	100.7			15	15.4	102.9	
α-terpinene						marjoram Soria	1	1.0	100.0	10.9
							3	3.2	107.9	
							5	5.0	100.0	
							10	10.1	101.3	
							15	15.0	100.0	
α-terpineol	marjoram 1	1	0.8	85.5	7.9	marjoram Sevilla	1	1.0	100.0	14.0
		3	3.2	105.7			3	2.8	92.7	
		5	5.4	108.3			5	5.1	101.9	
		10	10.1	100.5			10	10.6	106.2	
		15	15.3	101.8			15	14.9	99.4	
	marjoram 2	1	0.9	94.7	8.5	marjoram Soria	1	1.0	100.0	7.2
		3	3.2	105.3			3	3.1	104.3	
		5	5.3	105.6			5	5.3	106.7	
		10	10.6	106.2			10	10.3	102.5	
		15	14.4	96.0			15	15.0	100.0	
	marjoram 3	1	0.9	93.5	5.4					
		3	3.1	104.8						
		5	4.9	99.3						
		10	9.8	97.8						
		15	16.0	106.3						

Table 2. (Continued)

analyte	real sample	concn (μg mL^{-1}) added	concn (μg mL^{-1}) found	recovery (%)	concn in real sample (μg mL^{-1})	real sample	concn (μg mL^{-1}) added	concn (μg mL^{-1}) found	recovery (%)	concn in real sample (μg mL^{-1})
terpinolene	marjoram 1	1	0.9	94.7	ND[a]	marjoram Sevilla	1	1.2	113.3	0.3
		3	2.7	88.7			3	2.9	96.9	
		5	5.3	105.3			5	5.1	101.0	
		10	10.4	104.1			10	10.0	100.0	
		15	15.5	103.3			15	16.0	106.5	
	marjoram 2	1	0.8	80.7	1.6	marjoram Soria	1	1.0	100.0	4.0
		3	3.1	101.1			3	3.2	105.2	
		5	5.5	109.0			5	5.5	109.3	
		10	10.5	105.2			10	10.6	105.6	
		15	14.3	95.5			15	14.6	97.6	
	marjoram 3	1	0.9	98.0	2.0					
		3	3.0	100.0						
		5	4.9	99.3						
		10	10.5	105.5						
		15	15.0	100.0						
p-cymene	marjoram 1	1	0.8	81.8	ND	marjoram Sevilla	1	0.9	98.2	ND
		3	2.6	87.3			3	2.7	90.6	
		5	5.1	101.9			5	4.7	94.9	
		10	10.8	107.6			10	10.0	100.0	
		15	15.2	101.0			15	15.3	102.1	
	marjoram 2	1	0.9	89.5	0.3	marjoram Soria	1	0.8	81.8	2.5
		3	3.1	101.1			3	3.1	101.5	
		5	4.9	97.6			5	5.5	109.4	
		10	10.5	105.0			10	9.9	99.3	
		15	14.7	97.9			15	14.1	94.3	
	marjoram 3	1	0.8	83.5	0.8					
		3	3.1	104.3						
		5	5.5	109.9						
		10	10.0	100.0						
		15	15.1	100.4						

[a] ND, not detected.

found in real samples at this level of concentration. To validate the proposed analytical method, the marjoram samples were spiked with a standard mixture. A 10 mL volume of each solution of marjoram sample (100 μg mL^{-1}) was spiked at five different concentration levels, 1, 3, 5, 10, and 15 μg mL^{-1}, for each monoterpene. The results are summarized in **Table 2**, showing recoveries between 80 and 113%, as was expected, because at lower concentration it was difficult to separate some monoterpenes that presented poor absorbance in the UV−visible zone. As one of the objectives of this work was to classify marjoram samples on the basis of the main chemotypes, for this purpose it is not necessary to identify all of the analytes in the samples analyzed. According to the results shown in **Table 2**, terpinen-4-ol, α-terpineol, and linalool were present in all samples. Terpinen-4-ol is the major component in samples M1, M2, M3, and M5, and α-terpineol is the most prominent in M4. **Figure 3** shows electropherograms of two real samples (M2 and M4) of essential oils. In these electropherograms it was observed that terpinen-4-ol is the major component in sample M2 (see **Figure 3A**), whereas α-terpineol was the major terpene in sample M4 (see **Figure 3C**). When sample M2 was spiked with different amounts of monoterpene alcohols and hydrocarbons, α-terpinene was not found because its peak was overlapped with other components (see **Figure 3B**). In the real sample M4, *p*-cymene, α-pinene, α-terpinene, and γ-terpinene were not found (see **Figure 3C**). When sample M4 was spiked with the analytes of interest, components such as α-pinene, α-terpinene, and γ-terpinene were not found because their peaks overlapped with other components present in the matrix of the real sample (see **Figure 3D**).

These results are of great importance to classify marjoram samples on the basis of their main chemotypes. Nykänen (*6*) found terpinen-4-ol as the most prominent component in essential oils of marjoram samples cultivated in Finland. This author reported that the investigations of the essential oils of marjoram have shown that the aroma composition varies according to the geographical origin of the plants. Oberdieck (*12*) analyzed essential oils from marjoram samples of various origins (Germany, France, Hungary, Portugal, Egypt, Turkey, and Romania), and the main terpene found in these samples was terpinen-4-ol. Other authors (*3*, *10*) found in essential oils from marjoram samples cultivated in Turkey that carvacrol was the major component. These investigations conclude that there are two chemotypes of marjoram oils: *cis*-sabinene hydrate/terpinen-4-ol type and carvacrol/thymol type. However, Charai et al. (*5*) reported that linalool was the major component found in marjoram oils, and they classified this plant as a linalool chemotype. The importance of the identification of this type of compounds is related with the information obtained to check if the marjoram oils were obtained by distillation of *M. hortensis* Moench (syn. *Origanum majorana* L.) or from other species (*3*). Marjoram samples (*M. hortensis* Moench) cultivated in the south of Brazil and the north of Spain presented similar electropherograms and could be considered to be of the terpinen-4-ol chemotype. Probably, both samples were cultivated under the same conditions of temperate climate and geographical region, whereas marjoram cultivated in the south of Spain was grown in a different geographical region with a very hot and arid climate.

Conclusions. The aim of this paper was to demonstrate the uselfuness of the method previously developed by the authors to separate monoterpenes in real samples. Despite sample matrices that were very complex, separations and quantification of some monoterpene alcohols and hydrocarbons present in these

real samples were carried out. The electrophoretic method developed was applied for the identification and quantification of at least eight monoterpenes in this type of matrix for the first time. Moreover, it should be applied to other essential oils that have other types of monoterpenes. The use of this capillary electrophoresis technique for the analysis of marjoram samples can be considered as a new alternative to gas chromatography, the classical technique used at this time.

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